

# UNCLASSIFIED

AD NUMBER
ADB257105
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Sep 99. Other requests shall be referred to US Army Medical Research and Materiel Comd., Fort Detrick, MD 21702-5012.
AUTHORITY
USAMRMC ltr, 21 Feb 2003

THIS PAGE IS UNCLASSIFIED

AD \_\_\_\_\_

Award Number: DAMD17-98-1-8476

TITLE: Repression of the Androgen Receptor by WT1, a Tumor  
Suppressor Gene

PRINCIPAL INVESTIGATOR: Gail C. Fraizer, Ph.D., MPH

CONTRACTING ORGANIZATION: University of Texas/M.D. Anderson  
Cancer Center  
Houston, Texas 77030

REPORT DATE: September 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government  
agencies only (proprietary information, Sep 99). Other requests  
for this document shall be referred to U.S. Army Medical Research  
and Materiel Command, 504 Scott Street, Fort Detrick, Maryland  
21702-5012.

The views, opinions and/or findings contained in this report are  
those of the author(s) and should not be construed as an official  
Department of the Army position, policy or decision unless so  
designated by other documentation.

20000818 152

DMIC QUALITY IMPROVED 4

# NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

## LIMITED RIGHTS LEGEND

Award Number: DAMD17-98-1-8476

Organization: University of Texas/M.D. Anderson Cancer Center

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

Niningha Ocean Mtns  
07/27/00

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE September 1999		3. REPORT TYPE AND DATES COVERED Annual (15 Aug 98 -14 Aug 99)
4. TITLE AND SUBTITLE: Repression of the Androgen Receptor by WT1, a Tumor Suppressor Gene			5. FUNDING NUMBERS:  DAMD17-98-1-8476	
6. AUTHOR(S) Gail C. Fraizer, Ph.D., MPH				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Texas/M.D. ANDERSON  Houston, Texas 77030 E-MAIL: <a href="mailto:gfraizer@odin.mdacc.tmc.edu">gfraizer@odin.mdacc.tmc.edu</a>			8. PERFORMING ORGANIZATION REPORT NUMBER:	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES):  U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER:	
11. SUPPLEMENTARY NOTES This report contains colored photos				
Distribution authorized to U.S. Government agencies only proprietary information, Sep 99). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) The androgen pathway is central to prostate tumorigenesis. An increased risk of higher stage, more aggressive prostate cancer is associated with a more active androgen receptor (AR). We are investigating an innovative transcription based mechanism that represses AR activity <i>in vitro</i> : Our hypothesis is that the tumor suppressor gene, WT1, may play a role in prostate tumorigenesis mediated by repression of AR gene expression. To validate our AR promoter data we demonstrated that AR target gene down-regulation by WT1 is dependent on an intact DNA binding domain, is mediated by AR and is hormone dependent. Additionally we confirmed our RNA studies showing that WT1 protein expression patterns are inversely related to AR expression. Androgen responsive cell lines express AR but fail to express WT1, while androgen independent lines express WT1 and lack AR, suggesting a correlation with late-stage androgen independence. Recently we established stable transfected tumor cell lines and are now determining their growth characteristics with the intent of using them to establish a mouse model of prostate cancer progression. With the correlation of WT1 expression with higher-grade disease and the potential to demonstrate WT1 repression of AR expression in mice, we will establish the role of WT1 in the development of androgen independence.				
14. SUBJECT TERMS  Prostate Cancer			15. NUMBER OF PAGES 16	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT: Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE: Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT  Unlimited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

\_\_\_ Where copyrighted material is quoted, permission has been obtained to use such material.

\_\_\_ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

\_\_\_ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Gail Fraizer      10-7-99  
PI - Signature      Date

## TABLE OF CONTENTS

SF 298	2
Foreword	3
Table of Contents	4
Introduction	5
Body	5-10
Key Research Accomplishments	11
Reportable Outcomes	11
Conclusions	12
References	12
Appendices	14-15

## Introduction

The androgen pathway is central to prostate tumorigenesis. An increased risk of higher stage, more aggressive prostate cancer is associated with the inheritance of a more active androgen receptor (AR) (1-3). We are investigating an innovative transcription based mechanism that represses AR activity *in vitro*. The tumor suppressor gene, WT1, transcriptionally represses many growth control genes including AR. Our hypothesis is that WT1 may play a role in prostate tumorigenesis mediated by repression of AR gene expression. We have previously demonstrated that WT1 repression of the AR gene promoter construct (4) is mediated by DNA binding. To validate these exogenous AR promoter data we demonstrated that WT1 represses the endogenous AR promoter in androgen responsive cells (5). Now we have demonstrated that this AR target gene down-regulation by WT1 is dependent on an intact DNA binding domain, is mediated by AR and is hormone dependent. Additionally using Western blot analysis we confirmed the inverse relation between expression of WT1 and AR in prostate cancer cell lines, previously demonstrated by RT-PCR analysis. Androgen responsive cell lines express AR but fail to express WT1, while androgen independent lines express WT1 and lack AR, suggesting a correlation with late-stage androgen independence. Thus, our hypothesis that WT1 transcriptionally represses AR gene expression in WT1-producing prostate tumor cells is being tested using both *in vitro* and *in vivo* approaches which assess both the significance of WT1 expression in prostate tumors and the mechanism of AR repression. With our recent establishment of stably transfected LNCaP and MDAPCa2b lines we will now be able to determine their growth characteristics with the intent of using them to establish a mouse model of prostate cancer progression. If the correlation of WT1 expression with higher grade disease is upheld in on-going studies and if WT1 represses AR expression *in vivo*, then we will have established a model of WT1-mediated progression of prostate tumors to androgen independence.

## BODY OF WORK

We have demonstrated an inverse correlation between WT1 and AR expression in several prostate tumor cell lines, suggesting that WT1 may play a role in prostate tumorigenesis. This hypothesis is supported by previous observations by others that in prostate tumor biopsies a significantly higher percentage of tumor cells (30%) expressed WT1 protein than did adjacent normal cells (12%) and normal prostate biopsies showed no significant WT1 expression (6). Our recent work (discussed below) has confirmed and extended these previous findings.

## PREVIOUS RESULTS:

### 1. WT1 and AR expression are inversely correlated

The expression of AR during fetal and postnatal development in androgen target tissues is inversely correlated to WT1 expression. Using RT-PCR we previously confirmed this inverse relationship of WT1 and AR expression in the prostate, an androgen responsive tissue. In collaboration with Dr. Nora Navone, we determined that WT1 is not expressed in LNCaP and MDAPCa2b, two androgen-responsive prostate tumor cell lines expressing AR mRNA. Conversely, WT1 is expressed in DU145 and PC3; two androgen unresponsive, highly tumorigenic prostate tumor cell lines that lack AR expression. If WT1 also represses the AR gene promoter *in vivo*, then inhibition of AR-induced transactivation of AR target genes may suppress tumor cell growth in androgen-responsive prostatic tumor cells. This work has been extended by examining protein expression levels in the prostate tumor cell lines and in the tumor progression cell lines derived from both LNCaP and PC3 prostate cancer cell lines.

**2. WT1 repression of the AR promoter is mediated by DNA binding**

In previous work we have demonstrated 3-10-fold repression of the AR promoter by overexpression of WT1(-KTS) isoforms in transient transfection assays in HeLa, T47D (Breast cancer), SaOS (osteosarcoma), 293 (kidney) and TM4 (Sertoli) cells. The specificity of this repression was confirmed by demonstrating the inability of a zinc-finger mutant WT1 expression construct to repress the AR promoter construct in HeLa cells. Thus, WT1 repression of the AR promoter requires a functionally intact DNA binding domain. This repression was demonstrated by electrophoretic mobility shift assay (EMSA) to be mediated by binding of the WT1 protein to at least two regions containing 6 potential WT1 binding sites in the AR promoter. This work has been extended by searching for other possible mechanisms of interaction between WT1 and AR, such as protein-protein interactions. We co-transfected both AR and WT1 expression constructs into HeLa and Cos cells and examined the effect on downstream targets as well as directly assessing physical interactions by co-labeling studies.

**3. WT1 repression of the AR pathway**

For a better understanding of the role played by WT1 in regulating the expression of AR and AR-target genes, we examined the effect of overexpression of WT1 in 293 kidney and TM4 Sertoli cells expressing AR. In these assays the ability of WT1 to directly repress the endogenous AR promoter resulted in an indirect repression of an AR-target gene construct containing four copies of the ARE binding site (GGTACAnnnTGTTCT), the E1B TATA box and the luciferase gene. WT1 over-expression in 293 and TM4 cells down-regulates AR and indirectly results in a dose-dependent reduction in ARE-activated luciferase activity. These experiments have been extended to prostate tumor cells, LNCaP and MDAPCa2b. This has allowed us to confirm this indirect mechanism by dual labeling immunofluorescence assays of WT1 and AR expression in LNCaP cells transiently transfected with WT1. We are working towards reproducing these experiments in both LNCaP and MDAPCa2b in such a way that will allow quantitative western blot analysis of WT1 and AR expression in the transfected LNCaP and MDAPCa2b cells. Towards this end we have stably transfected a subline of LNCaP cells (obtained from our collaborator, Dr. G. Jenster) with WT1-FLAG expression constructs (obtained from another collaborator, Dr. C. Roberts). The stably transfected lines will allow quantitative western blot analysis using both WT1 and FLAG Ab. Stable transfections of the MDAPCa2b cell line are also being performed in collaboration with Dr. Navone. Concurrently we have established a TET-ON stably transfected LNCaP cell line in which we have transiently transfected WT1-FLAG expression constructs.

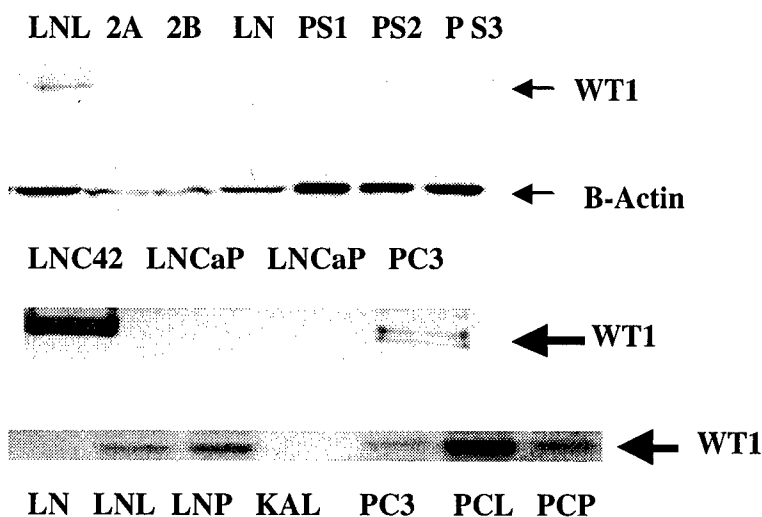
These previous results demonstrated that: 1) WT1 and AR expression are inversely correlated in androgen target tissues, 2) WT1 protein binds at least two of the WT1 binding sites in the AR promoter in vitro, 3) WT1 directly binds and represses exogenously added AR promoter constructs in kidney and gonadal cells, and 4) WT1 repression of the endogenous AR promoter in kidney and gonadal cells interferes with the androgen signal transduction pathway causing down-regulation of AR target gene transcription. In the past year these findings of inverse expression and downregulation of AR by WT1 have been confirmed in prostate cell lines.



**YEAR ONE RESULTS:**

**TECHNICAL OBJECTIVE I.** To determine the prognostic significance of WT1 expression in prostate tumors.

**WT1 protein is strongly expressed in androgen insensitive prostate cancer cell lines , but not in androgen sensitive prostate cancer cell lines.**



Nuclear extracts containing 30 ug of protein were separated by electrophoresis through a 12% SDS-polyacrylamide gel and analyzed by Western blotting using WT1 Ab (c19, Santa Cruz). Immunoreactive proteins were visualized by ECL-Plus luminescence and autoradiography. High salt extracts were prepared from the following cell lines: ; LNL, LNCaP-LN3; 2A, MDAPCa2a; 2B, MDAPCa2b; LN, LNCaP; PS1, primary cultures of mouse stromal cells ; PS2; PS3; LNC42, LNCaP-C42; LNCaP; ; P, PC3; LNP, LNCaP-Pro5;

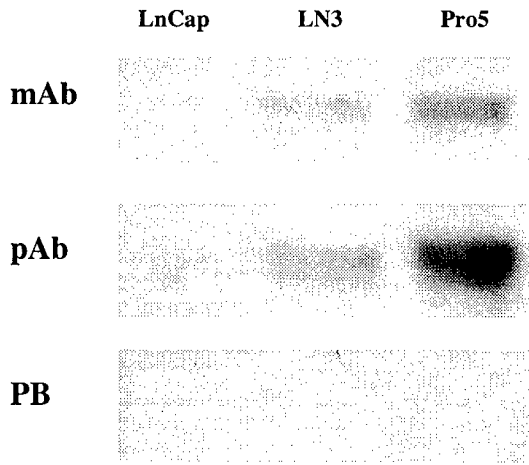
KAL, marker; ; PCL, PC3-LN; PCP, PC3-Pro. The position of the 45-47 kd WT1 proteins is marked with an arrow as is the position of the  $\beta$ -actin control. Note that WT1 expression is higher in the LNCaP sublines (LN3, Pro5 and C42) than in LNCaP.

These protein assays have now confirmed our previous RNA expression studies showing that WT1 expression patterns are inversely related to AR expression. Androgen responsive normal mouse prostate stromal lines and tumor cell lines express AR but fail to express WT1, while androgen independent lines express WT1 and lack AR. The androgen independent LNCaP-C42 and LN-LN3 variants express WT1, while the androgen dependent LNCaP, MDAPCa2a and MDAPCa2b cells lack WT1, suggesting a correlation of WT1 expression with late-stage androgen independence.

Our results are in agreement with those showing primary cultures of normal epithelial cell strains lack significant WT1 expression (7). However, our results do not support the findings of others that some normal prostate stromal cell strains do express WT1, as detected by RT-PCR and RNase PA (7). In our primary cultures of mouse stromal cells, we saw no evidence of WT1 expression but did observe androgen response and strong AR expression. Similarly, the reported lack of WT1 expression in epithelial tumor cell strains, but presence in stromal tumor cell strains (7), is not supported by our immunohistochemistry results (see appendix). Indeed, the original finding was surprising as high WT1 expression levels seen in nephroblastomas are restricted to epithelial predominant tumors and are reduced in stromal predominant tumors (8). We have now observed WT1 expression in several epithelial prostate tumor cell lines, particularly the more aggressive, androgen unresponsive lines.

Interestingly LNCaP-Pro5, previously reported to be androgen sensitive (9), does express some WT1. However, using a dual labelling assay we have developed for WT1 and AR co-expression studies using TRITC-anti-rabbit and FITC anti-mouse antibodies we observed an inverse relation of WT1 to AR in these cells as well (Fig 12, Appendix). LNCaP cells express AR (FITC staining in left panel) and

5nM R1881 strongly induces AR immunofluorescence in LNCaP (FITC staining in right panel) But no WT1 staining is observed. In contrast LNCaP-Pro5 cells express AR only weakly (FITC staining in left panel) but do express WT1 in some cells (TRITC staining in left panel). Interestingly, in our hands, this androgen sensitive line is not highly responsive to R1881 induction. While 5nM R1881 strongly



induces AR immunofluorescence in LNCaP it only weakly induces AR in LNCaP-Pro5. (FITC staining in right panel). However, the AR positive LNCaP-Pro5 cells lack WT1 and the WT1 positive LNCaP-Pro5 cells lack AR expression. Thus, AR and WT1 are not co-expressed in the same cells as shown by the lack of yellow staining with the dual staining procedure. We confirmed these results by western blot analysis of LNCaP, LNCaP-LN3, and LNCaP-Pro5 progression lines. Initially we probed the filter with a mouse mAb (DAKO) which detects an amino terminus peptide of WT1. Then we stripped the western blot and reprobed with a polyclonal Ab which detects a carboxy terminus peptide. Finally we stripped and reprobed the western

blot with the polyclonal Ab which had been preincubated with blocking peptide (the immunogen). The mAb and the pAb gave identical results and the pAb was completely blocked by preincubation with the peptide. Thus, unlike LNCaP cells, the more aggressive LNCaP sublines express WT1, although on a single cell level, WT1 expression is inversely related to AR.

**WT1 protein is expressed in both nucleus and cytoplasm of androgen insensitive prostate cancer cell lines.** Western blot analysis of nuclear extracts and cytoplasmic remnants (supernatant removed from nuclear pellets) showed the presence of WT1 protein in both the nuclear and cytoplasmic fraction of androgen insensitive cell lines (DU145, LNCaP-LN3, PC3, and PC3 sublines). We confirmed the cytoplasmic location of WT1 protein using the same polyclonal anti-WT1 Ab (C19, Santa Cruz) in immunohistochemical analyses of prostate epithelial cell lines grown in 8-well chamber slides. We observed dark brown staining nuclei and light brown staining cytoplasm in the androgen insensitive cell lines (Data not shown). Specificity of staining was confirmed by treating Ab with WT1 blocking peptide prior to incubation of the polyclonal Ab with cell lines. While WT1 expression is not limited to the nucleus, its function (if any) in the cytoplasm is unknown.

#### **WT1 protein is focally expressed in both nucleus and cytoplasm of prostate tumor sections**

Using anti-WT1 Ab (C19, Santa Cruz) both dark brown staining nuclei and light brown staining cytoplasm are visible in prostate tumor epithelial cells (**Fig 6A** in appendix). **Fig 6B.** Focal cytoplasmic staining of epithelial cells of high grade prostate tumor sections. **Fig 6C.** Right panel shows specificity of staining by treating Ab with WT1 blocking peptide prior to incubation with tissues.

Stage and grade of prostate tumors were determined by medical record examination and correlated with WT1 expression. In limited analyses of 10 patients, WT1 expression correlates with high grade and stage tumors (**Fig. 7**). In samples of Gleason 5 grade tumors (**Fig 7C**) we observed no WT1 expression, while in Gleason 9 grade sections some displayed focal epithelial cytoplasmic staining (**Fig 7B shows high power view of Fig 7A**), sometimes accompanied by nuclear staining but often not. Normal prostate tissues and low grade PIN samples lacked WT1 expression. Additional evidence of correlation with grade using the TRAMP mouse model (10) confirmed these findings (**Fig**

8). WT1 expression was absent in normal tissues and PIN of all grades (**Fig 8C**), but was present in poorly differentiated mouse tumors (**Fig 8B**). Left panel (**Fig 8A**) shows specificity of staining by treating Ab with WT1 blocking peptide prior to incubation with tissues. As documentation of protein expression is more relevant to WT1 function we have focused our expression studies on immunohistochemistry and western blot assays. If unexpected isoform ratios are observed in mRNA isolated from cultured prostate tumor cells, then we will obtain frozen prostate tumor tissue for RNA analysis.

**TECHNICAL OBJECTIVE II.** To determine whether WT1 represses the androgen signal transduction pathway in prostate tumor cell lines.

Initially this work was extended by searching for other possible mechanisms of interaction between WT1 and AR, such as protein-protein interactions. We co-transfected both AR and WT1-FLAG expression constructs into HeLa and Cos cells and examined the effect on downstream targets as well as directly assessing physical interactions by immunoprecipitation assays (not shown) and co-labeling studies using immunofluorescent antibodies. Using a dual labelling assay we have developed for WT1 and AR co-expression studies we incubated the cotransfected monolayers (grown on glass slides) with monoclonal anti-FLAG Ab and polyclonal anti-AR Ab followed by incubation with TRITC-anti-mouse and FITC anti-rabbit antibodies. We detected TRITC-labeled and FITC-labeled cells and some dually-labeled cells (**Fig 11**, Appendix), however, on high power we observed primarily adjacent labelling without direct overlapping (as detected by the yellow color in far right panel).

Recently these dual labelling experiments have been extended to androgen responsive prostate tumor cells, LNCaP and MDAPCa2b (**Fig 12**). This has allowed us to exclude direct physical interaction and supports our earlier data suggesting an indirect mechanism of endogenous AR repression by WT1. Using dual labeling immunofluorescence assays of WT1 and AR expression in LNCaP cells transiently transfected with WT1 we also demonstrated inverse expression. We are working towards reproducing these experiments in both LNCaP and MDAPCa2b in such a way that will allow quantitative western blot analysis of WT1 and AR expression in the transfected LNCaP and MDAPCa2b cells. Towards this end we have stably transfected a subline of LNCaP cells (obtained from our collaborator, Dr. G. Jenster) with WT1-FLAG expression constructs (obtained from our collaborator, Dr. C. Roberts). These include the normal (-)KTS isoforms and the DDS zinc finger mutant (-)KTS isoforms which fail to bind DNA. The stably transfected lines will allow quantitative western blot analysis using both WT1 and FLAG Ab. Transfection efficiency in LNCaP cells is so low as to preclude bulk cell analysis of transfected monolayers. Thus we used a dual labelling FITC/TRITC Ab based assay to observe rare individual cells which were transfected with WT1 and were induced by hormone treatment.

Stable transfections of the MDAPCa2b cell line are also being performed in collaboration with Dr. Navone. Concurrently we have established a TET-ON stably transfected LNCaP cell line in which we have transiently transfected WT1-FLAG expression constructs (obtained from our collaborator, Dr. C. Roberts).

**TECHNICAL OBJECTIVE III.** Establish a mouse model for prostate cancer progression.

With our recent establishment of stably transfected lines constitutively expressing WT1 or the zinc finger mutants we will now be able to determine their growth characteristics with the intent of using them to establish a mouse model of prostate cancer progression. Towards this end we have established several stably transfected LNCaP cell lines. We have established a TET-ON stably transfected LNCaP cell line which we are able to transiently transfect with WT1-FLAG expression

constructs (obtained from our collaborator, Dr. C. Roberts). However, transfection efficiency in LNCaP cells is so low as to preclude selection of dually transfected monolayers. Thus the TET-On LNCaP cell lines which we have established respond to hormone but have not survived TRE-WT1 co-transfection with the TK-hygromycin selection construct. We are currently using an alternative drug selection marker (puromycin), but have also used an alternative approach to establishing stable lines. Thus we have established non-inducible WT1-FLAG tagged stably transfected LNCaP cells and stable transfections of the MDAPCa2b cell line are also being performed in collaboration with Dr. Navone. Because we anticipate poor growth of these lines (due to preliminary comparisons of FLAG vector transfected vs FLAG-WT1 transfected growth characteristics) we are also continuing our efforts in establishing regulatable WT1 expressing lines.

## **PLANS for YEAR TWO**

**TECHNICAL OBJECTIVE III.** Establish a mouse model for prostate cancer progression

- Task A. Assess growth characteristics of four stably transfected prostate tumor cell lines (months 12-18). Inducible overexpression of WT1 is expected to result in altered growth and tumorigenic characteristics.
- Task B. Each stably transfected prostate tumor cell line will be injected into nude mice to determine whether growth suppression in vitro correlates with tumor suppression or growth in vivo (18-24). We expect that if WT1 expression correlates with higher grade disease and AR repression, then it may play a role in the selection of androgen independent prostate tumor cells.

## KEY RESEARCH ACCOMPLISHMENTS

- **WT1 protein is strongly expressed in androgen insensitive prostate cancer cell lines , but not in androgen sensitive prostate cancer cell lines.**
- **WT1 protein is focally expressed in both nucleus and cytoplasm of prostate tumor sections**
- **WT1 expression correlates with high grade human tumors, poorly differentiated TRAMP mouse tumors.**
- **Normal prostate tissues and low grade PIN samples lacked WT1 expression in both human and mouse sections.**
- **TET-ON stably transfected LNCaP and WT1 stably transfected LNCaP and MDAPCa2b cell lines have been established.**

## REPORTABLE OUTCOMES

### **Presentations:**

REGULATION OF THE ANDROGEN RECEPTOR BY A TUMOR SUPPRESSOR GENE, WT1.  
Gail Fraizer. Fifty-first Annual Symposium on Fundamental Cancer Research, Poster Discussion Session II, Houston, Oct 28, 1998

REGULATION OF THE ANDROGEN RECEPTOR BY A TUMOR SUPPRESSOR GENE, WT1.  
Gail Fraizer. New Research Approaches in the prevention and cure of Prostate Cancer, AACR Special Conference, Dec, 1998

REGULATION OF THE ANDROGEN RECEPTOR BY A TUMOR SUPPRESSOR GENE, WT1.  
Gail Fraizer. American Urological Association Annual Meeting, Dallas, Texas, 1999

ROLE OF WT1 IN PROSTATE CANCER PROGRESSION, Urology Department Seminar Series,  
July 7, 1999

### **Published Abstracts:**

Annamaria Zaia, Ryuji Shimamura, Michael Badzioch, Grady Saunders, **Gail Fraizer**.  
Regulation of the Androgen Receptor by a Tumor Suppressor gene, WT1. AACR Special Conference in Cancer Research Abstract A#8, Indian Wells, CA, (1998)

Annamaria Zaia, Ryuji Shimamura, Grady Saunders, **Gail Fraizer**,  
Regulation of the Androgen Receptor by a Tumor suppressor gene, WT1. AUA 1999 Annual meeting, Dallas, Texas, 1999

### **Development of Cell Lines**

TET-ON stably transfected LNCaP cell lines have been established, clone #37 has been distributed to collaborators for use in other projects.

## CONCLUSIONS

If WT1 transcriptionally represses AR gene expression in WT1-producing prostate tumor cells, two possible predictions can be made. 1) Overexpression of a tumor suppressor and subsequent repression of the AR pathway could lead to tumor suppression. Or 2) If suppression of AR confers a growth advantage selecting AI tumor cells, then we would predict an outgrowth of aggressive hormone refractory tumor cells causing tumor progression. In the latter case, growth advantages conferred by the repression of the AR by WT1 lead to androgen independence. The hypothesis that WT1 can mediate androgen independent tumor progression is being tested using both in vitro and in vivo approaches which will assess both the significance of WT1 expression in prostate tumors and the mechanism of AR repression. The first two specific aims have been performed concurrently and results will be confirmed and extended in the next few months. We will continue to examine WT1 expression for correlation with higher grade disease in an expanded analysis of metastatic human tumors and post-castration TRAMP mouse tissue sections. Stably transfected LNCaP and MDAPCa2b lines will be evaluated for their growth characteristics with the intent of using them to establish a mouse model of prostate cancer progression in the second year of this proposal. Additionally we will determine whether any tumors established by stably transfected LNCaP lines are androgen independent, ie, does WT1 also repress AR expression in vivo. Together these results will support our hypothesis that WT1 plays a role in the progression of prostate tumors to androgen independence.

## References

1. M. Badzioch, Edwards, S.M., Amos, C., Pack, R., Dearnaley, D.P., Arden-Jones, A., Easton, D.F., Ford, D., Shearer, R., Dowe, A., Minter, R., Collins, N., Saunders, G.F., and Eeles, R.A. Androgen receptor-CAG trinucleotide repeat length as a risk factor. (1997) Annual Meeting of the American Association of Cancer Research (Abstract #1434)
2. Giovannucci, E., Stampfer, M.J., Krithivas, K., Brown, M., Brufsky, A., Talcott, J., Hennekens, C.H., and Kantoff, P.W. (1997) Proc. Natl. Acad. Sci. USA 94, 3320-3323
3. Jenster, G., deRuiter, P.E., vanderKorput, H., Kuiper, G., Trapman, J., and Brinkmann, A.O. Changes in the abundance androgen receptor isotypes: effects of ligand treatment, glutamine-stretch variation, and mutation of putative phosphorylation sites. (1994) Biochem. 33:14064-14072.
4. Shimamura, R., Fraizer, G.C., Trapman, J., Lau, Y.C., and Saunders, G.F. The Wilms' tumor gene WT1 can regulate genes involved in sex determination and differentiation: SRY, Mullerian inhibiting substance, and androgen receptor. (1997) Clinical Cancer Research 3:2571-2580.
5. Zaia, A., Fraizer, G.C., Piantanelli, L., Saunders, G.F. Wilms Tumor suppressor gene WT1 regulates androgen-signaling pathway by repression of androgen receptor gene transcription. Biochemical Biophysica Acta
6. Ng, N.G., Tenp, S.P., Bales, W., Rodeck, U., and Veltei, R.W. (1995) Increased extension of Wilms' tumor protein (WT1) in prostate cancer.
7. Dong, G, Rajah, R, Vu, T, Hoffman AR, Rosenfeld, RG, Roberts, CT, Jr., Peehl, DM and Cohen P. Decreased expression of Wilms' tumor gene WT1 and elevated expression of insulin growth factor-II (IGF-II) and type 1 IGF receptor genes in prostatic stromal cells from patients with benign prostatic hyperplasia. (1997) J. Clin. Endo and Metabolism 82, 2198-2203.
8. Miwa, H., Tomlinson, G.E., Timmons, C.S., Huff, V., Cohn, S.L, Strong, L.C., and Saunders, G.F. (1992) RNA expression of the WT1 gene in Wilms' tumors in relation to histopathology and therapy. J. Nat'l Cancer Inst. 84:181-187.

9. Pettaway CA, Pathak,S., Greene, G., Ramirez, E., Wilson, M., Killion, J., Fidler, IJ (1996) Selection of Highly Metastatic Variant of Different Human Prostatic Cancinomas Using Orthotopic Implantation in Nude Mice. Clin. Cancer Res. 2:1627-1636,
- 10.Greenberg, NM, DeMayo, F, Finegold, MJ, Medina, D, Tilley, WD, Aspinal, JO, Cunha, GR, Donjacour AA, Matusik, RJ, Rosen J (1995) Prostate Cancer in a transgenic mouse. Proc Natl Acad Sci USA 92:3439-3443.

FIG. 6

cytoplasmic and nuclear  
WT1 expression in  
prostate tumors



FIG. 7

WT expression in Gleason 9 prostate cancer

A.



B.



WT expression in Gleason 5 prostate cancer

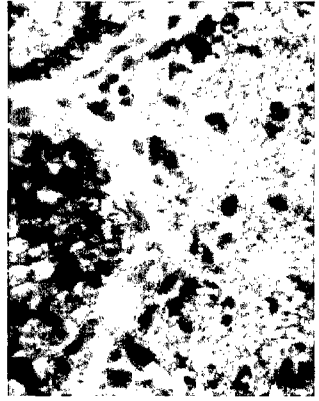
C.



FIG. 8

WT1 expression in TRAMP model: poorly differentiated tumor

A.



B.



WT1 Expression in TRAMP Model: PIN

C.





Fig. 11

Co-localization of WT1 and AR in co-transfected cos cells

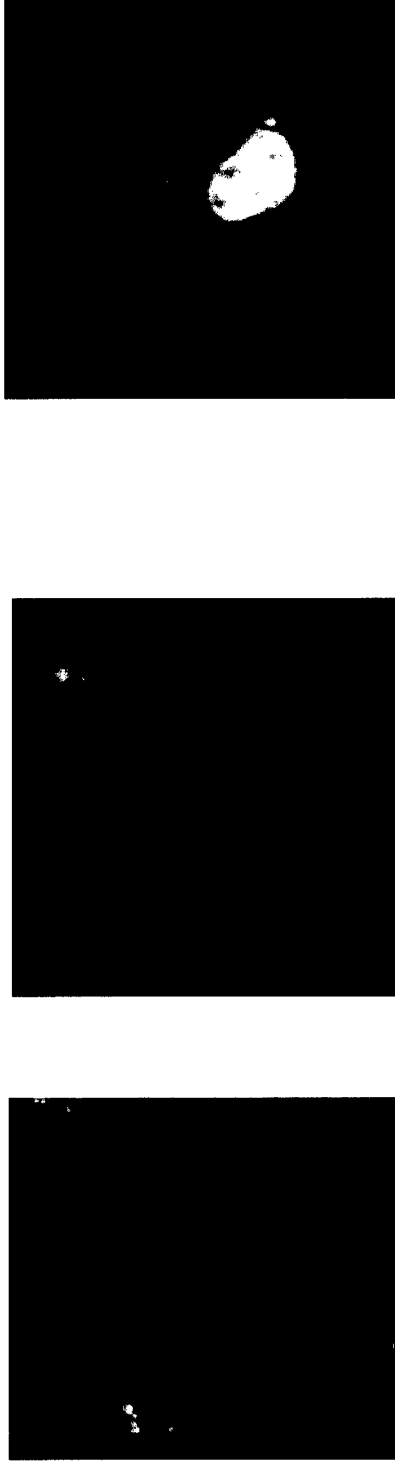


Fig. 12

Inverse expression of WT1 and AR in LNCap and LNCaP-Pro5 cells





DEPARTMENT OF THE ARMY  
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND  
504 SCOTT STREET  
FORT DETRICK, MARYLAND 21702-5012

REPLY TO  
ATTENTION OF:

MCMR-RMI-S (70-1y)

21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information  
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,  
VA 22060-6218

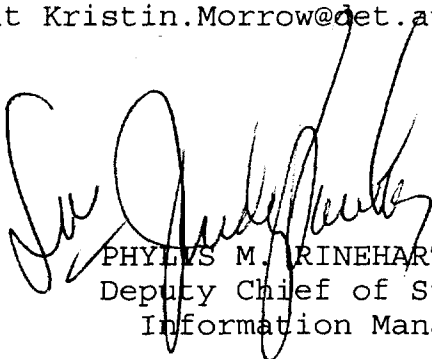
SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

  
PHYLLIS M. RINEHART  
Deputy Chief of Staff for  
Information Management

ADB263458	ADB282838
ADB282174	ADB233092
ADB270704	ADB263929
ADB282196	ADB282182
ADB264903	ADB257136
ADB268484	ADB282227
ADB282253	ADB282177
ADB282115	ADB263548
ADB263413	ADB246535
ADB269109	ADB282826
ADB282106	ADB282127
ADB262514	ADB271165
ADB282264	ADB282112
ADB256789	ADB255775
ADB251569	ADB265599
ADB258878	ADB282098
ADB282275	ADB232738
ADB270822	ADB243196
ADB282207	ADB257445
ADB257105	ADB267547
ADB281673	ADB277556
ADB254429	ADB239320
ADB282110	ADB253648
ADB262549	ADB282171
ADB268358	ADB233883
ADB257359	ADB257696
ADB265810	ADB232089
ADB282111	ADB240398
ADB273020	ADB261087
ADB282185	ADB249593
ADB266340	ADB264542
ADB262490	ADB282216
ADB266385	ADB261617
ADB282181	ADB269116
ADB262451	
ADB266306	
ADB260298	
ADB269253	
ADB282119	
ADB261755	
ADB257398	
ADB267683	
ADB282231	
ADB234475	
ADB247704	
ADB258112	
ADB267627	